Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 502 (2004) 47–58

www.elsevier.com/locate/ejphar

Differential effects of endogenous and synthetic cannabinoids on voltage-dependent calcium fluxes in rabbit T-tubule membranes: comparison with fatty acids

Murat Oz[a,*], Yulia Tchugunova[b], Meral Dinc[b]

[a]*National Institute on Drug Abuse, National Institutes of Health, DHHS, Intramural Research Program, Cellular Neurobiology Branch, 5500 Nathan Shock Drive, Baltimore, MD, 21224, USA*

[b]*Department of Pulmonary Diseases, Oncological Hospital, Ankara, 06947, Turkey*

Received 6 May 2004; received in revised form 9 August 2004; accepted 27 August 2004

Abstract

The effects of cannabinoid receptor ligands including 2-arachidonoylglycerol, R-methanandamide, Δ^9-THC (Δ^9-tetrahydrocannabinol), WIN 55,212-2 [4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6*H*-pyrrolo[3,2,1ij]quinolin-6-one], CP 55,940 ([1alpha,2beta-(*R*)-5alpha]-(−)-5-(1,1-dimethyl)-2-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl-phenol]) and a series of fatty acids on depolarization-induced Ca^{2+} effluxes mediated by voltage-dependent Ca^{2+} channels were investigated comparatively in transverse tubule membrane vesicles from rabbit skeletal muscle. Vesicles were loaded with $^{45}Ca^{2+}$ and membrane potentials were generated by establishing potassium gradients across the vesicle using the ionophore valinomycin. Endocannabinoids, 2-arachidonoylglycerol and R-methanandamide (all 10 μM), inhibited depolarization-induced Ca^{2+} effluxes and specific binding of [^{3}H]PN 200–110 (isradipine) to transverse tubule membranes. On the other hand, synthetic cannabinoids, including CP 55,940, WIN 55,212-2, and Δ^9-THC (all 10 μM), were ineffective. Additional experiments using endocannabinoid metabolites suggested that whereas ethanolamine and glycerol were ineffective, arachidonic acid inhibited Ca^{2+} effluxes and specific binding of [^{3}H]PN 200–110. Further studies indicated that only those fatty acids containing two or more double bonds were effective in inhibiting depolarization-induced Ca^{2+} effluxes and specific binding of [^{3}H]PN 200–110. These results indicate that endocannabinoids, but not synthetic cannabinoids, directly inhibit the function of voltage-dependent calcium channels (VDCCs) and modulate the specific binding of calcium channel ligands of the dihydropyridine (DHP) class.

Keywords: Calcium channel; Endocannabinoid; Cannabinoid; Fatty acid; Skeletal muscle

1. Introduction

Endocannabinoids have been defined as endogenously produced messenger molecules that bind to and activate cannabinoid receptors in a G-protein-dependent manner (Piomelli, 2003). Major endocannabinoids are derivatives of arachidonic acid, namely, *N*-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol. These molecules bind to cannabinoid CB_1 and/or CB_2 receptors and mimic the effects of synthetic cannabinoids in several in vitro preparations (Piomelli, 2003). However, several reports also indicate that endocannabinoids and the phytochemical Δ^9-THC (Δ^9-tetrahydrocannabinol) can produce effects that are not mediated by the activation of the cloned cannabinoid CB_1 and/or CB_2 receptors. For example, it has been demonstrated that endocannabinoids, such as anandamide and/or 2-arachidonoylglycerol, can inhibit the function of gap junctions (Venance et al., 1995), voltage-dependent Ca^{2+} channels (Oz et al., 2000; Chemin et al., 2001; Guo and Ikeda, 2004), Na^+ channels (Nicholson et al., 2003), various types of K^+ channels (Poling et al., 1996; Maingret et al., 2001), 5-HT_3 receptor function (Barann et al., 2002; Oz et al., 2002a), and nicotinic

* Corresponding author. Tel.: +1 410 550 6565; fax: +1 410 550 1621.
E-mail address: moz@intra.nida.nih.gov (M. Oz).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.08.052

acetylcholine receptors (Oz et al., 2003, 2004a). This suggests that additional molecular targets for certain classes of endocannabinoids and other lipid mediators exist, and that these targets may represent important sites for cannabinoids in the excitable cells.

In skeletal muscle fibers, cannabinoids have been shown to inhibit muscle contractions (Kumbaraci and Nastuk, 1980) and have been reported to have some ameliorating effects on muscle spasm induced by excessive neuronal activity such as in muscular dystrophy (Baker and Pryce, 2003). Voltage-dependent calcium channels (VDCCs) play functional roles during tetanic contractions and maintained spasm-like contractures in both mammalian and amphibian skeletal muscles (Oz and Frank, 1991; Oz et al., 1992). Recently, the effects of anandamide on the function of VDCCs were investigated on depolarization-induced Ca^{2+} fluxes mediated by L-type VDCCs in purified T-tubule membranes (Oz et al., 2000), and it was found that anandamide significantly inhibits the function of VDCCs in a cannabinoid receptor-independent manner.

There are four different classes of cannabinoid receptor ligands that are employed currently in pharmacological research. These include the classical cannabinoids, typified by the phytochemical Δ^9-THC (Δ^9-tetrahydrocannabinol); the nonclassical cannabinoids, typified by the agonist CP 55,940 [[1alpha,2beta-(*R*)-5alpha]-(−)-5-(1,1-dimethyl)-2-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl-phenol]; the aminoalkylindoles, such as WIN 55,212-2 [4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6*H*-pyrrolo [3,2,1ij]quinolin-6-one]; and the arachidonic acid derived eicosanoid molecules such as arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (Howlett et al., 2002). The present study was performed to compare the effects of a range of cannabinoid receptor ligands representing each of these classes and to investigate the structural determinants of the effects of endocannabinoids on depolarization-induced Ca^{2+} fluxes mediated by L-type VDCCs in purified T-tubule membranes.

2. Materials and methods

2.1. Preparation of transverse tubule membranes

The back and hind muscles of small (1-1.5 kg) New Zealand white rabbits were used to prepare microsomal membranes and T-tubules were isolated by sucrose gradient centrifugation as described previously (Dunn, 1989). The animals were cared for in accordance with the principles and guidelines of the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 86-23, revised 1985). T-tubule membranes were resuspended and equilibrated in low potassium buffer (10 mM HEPES-Tris, pH 7.4, 145 mM choline chloride, 5 mM potassium gluconate, 0.02% NaN_3) and stored at −86 °C. Prior to use, the vesicles were subjected to freeze–thaw cycles to equilibrate intracellular and extracellular ions (Dunn, 1989; Oz et al., 1993).

2.2. $^{45}Ca^{2+}$ efflux assay

Approximately 0.4 mg/ml of membrane vesicles were loaded with $^{45}Ca^{2+}$ by the addition of one-half volume of isotopically diluted $^{45}CaCl_2$ solution in the same buffer to give a final concentration of 5 mM total Ca^{2+} containing approximately 50 μCi/ml $^{45}Ca^{2+}$ (ICN, Irvine, CA, USA). After two freeze–thaw cycles to load the vesicles with Ca^{2+}, the suspensions were kept on ice until use, which was usually within 1–2 h. A two-step filtration assay (Dunn, 1989) was used to investigate voltage-dependent $^{45}Ca^{2+}$ efflux. Briefly, 25 μl of loaded membranes were diluted first with 975 μl of high K^+ buffer (10 mM HEPES-Tris, pH 7.4, 120 mM potassium gluconate, 30 mM choline chloride, 0.133 mM EGTA) containing 0.1 μM valinomycin and where appropriate, the desired drug. This first dilution is designed to mimic the resting state of the cell by generating an outside negative membrane potential of −80 mV and to reduce the extravesicular (corresponding to intracellular in an inside–out vesicle) free Ca^{2+} to less than 100 nM. After a 10-min incubation at room temperature (18–21 °C), 0.9 ml was removed and applied to a GF/C filter, which had been pre-equilibrated in the same buffer and dried under a vacuum. Excess buffer was removed under a vacuum, and 1 ml of depolarizing buffer (10 mM HEPES-Tris, pH 7.4, 5 mM potassium gluconate, 145 mM choline chloride, 0.133 mM EGTA, 0.1 mM valinomycin) was added. This two-step procedure (F) is referred to as 5–120–5 mM K^+ in the text. Control experiments (C) were carried out using dilution buffers with constant K^+ concentration (5–5–5 mM K^+). Efflux was allowed to continue on the filter for 15 s and extravesicular solution was removed by rapid washing with two 5 ml volumes of a "stop" solution (10 mM HEPES-Tris, pH 7.4, 145 mM choline chloride, 5 mM potassium gluconate, 0.5 mM $LaCl_3$, 30 mM sucrose). Filters with their absorbed membrane vesicles were dried, extracted with 5 ml of Hydrofluor™ (National Diagnostics, Florida, USA) scintillation fluid and counted for $^{45}Ca^{2+}$.

Anandamide, R-methanandamide, and 2-arachidonoylglycerol were from Tocris Cookson (St. Louis, MO). All other chemicals were from Sigma (St. Louis, MO, USA). Δ^9-THC was provided by the National Institute on Drug Abuse (NIDA) Drug Supply System/National Institutes of Health (NIH), Baltimore, MD. Fatty acids were added from stock solutions in dimethylsulphoxide (DMSO) and, in order to minimize possible solvent effects, all samples, including controls, contained DMSO at the same final concentration (<0.2%). Fatty acids were obtained from following sources; 9,12-hexadecadienoic acid (16:2, Deva Biotech, Hatboro, PA), 9,12-octadecadienoic acid 11,14-eicosadienoic acid (Cayman Chemical, Ann Arbor, MI) 13,16-docosadienoic acid (22:2, Nu-Chek Prep, Elysian, MN) and other fatty acids were from Sigma. Stock solutions

of fatty acids were stored under N_2 atmosphere in the dark at −20 °C. Drugs and/or other agents were added as an ice-cold buffer solution (20 mM), and incubated with $^{45}Ca^{2+}$-loaded vesicles.

2.3. Binding studies

Experiments on the binding of (+)-[^{3}H]PN 200–110 (Isradipine, DuPont-New England, USA) were conducted similar to our previous studies (Dunn, 1989). All binding assays were carried out under subdued lighting to minimize ligand photolysis. Briefly, aliquots of membranes (100 μg) were added to different concentrations of radiolabeled ligand yielding a final concentration of 0.02 mg/ml T-tubule membranes in a total volume of 0.8 ml. After 60-min incubation at room temperature, 0.4 ml aliquots of each sample were filtered under a vacuum through Whatman GF/C filters and washed rapidly with 5 ml of ice-cold assay buffer. The filters were dried and extracted in 5 ml of Hydrofluor™ (National Diagnostics) scintillation fluid before counting for ^{3}H. Triplicate 50-μl samples of the incubation mixtures were counted directly for estimations of total binding. Nonspecific binding was estimated from parallel measurements of binding in the presence of 5 μM unlabeled nifedipine.

2.4. Data analysis

All data are expressed as the arithmetic means and standard errors of the means (S.E.M.) with the number of determinations (n) indicated. In each experiment, at least 5 determinations were made of the amount of $^{45}Ca^{2+}$ retained by the vesicles under control conditions, i.e., in the absence of any changes in membrane potential and in the absence of a drug. The mean counts per minute (cpm) was first calculated from control determinations and then normalized to 100%. The cpm of each determination was also normalized to the mean cpm to calculate the S.E.M. for each control. Data obtained under other control conditions are expressed as a percentage of control values. Statistical evaluation of data was made using analysis of variance (ANOVA) method. For data analysis and calculations, computer-fitting software Origin™ (Microcal Software, Massachusetts, USA) was used. The logistic function described below was used to calculate the IC_{50} and the slope factor (equivalent to Hill coefficient).

$$[Y] = \frac{A - B}{1 + \left(\frac{[L]}{[IC_{50}]}\right)^{n}} + B$$

where [Y] is the percentage of bound radioligand displaced in the presence of increasing concentrations of competing ligand [L], IC_{50} is the concentration of competing ligand causing 50% inhibition of the radioligand binding, n is the slope factor, A is the initial value, and B is the maximum value.

3. Results

3.1. Effect of endogenous and synthetic cannabinoids on depolarization-induced Ca^{2+} fluxes

A schematic depicting the orientation of the isolated T-tubule vesicles and an outline of the two-step protocol is illustrated in Fig. 1A. In the absence of changes in membrane potential (5–5–5 mM K^+, i.e., control conditions in which the K^+ concentration was constant at 5 mM throughout the flux assay), there was no efflux of $^{45}Ca^{2+}$ from the T-tubule vesicles (C in inset to Fig. 1A). After repolarization by addition of high external K^+, subsequent exposure to depolarizing conditions for 15 s (5–120–5 mM K^+, F in inset to Fig. 1A) reduced the $^{45}Ca^{2+}$ content of the vesicles to approximately 30–35% of control values.

Following a 10-min incubation, endocannabinoids (10 μM), 2-arachidonoylglycerol and R-methanandamide inhibited the flux responses significantly without affecting the content of $^{45}Ca^{2+}$ in the vesicles under control circumstances (P<0.05, ANOVA, n=7–8, Fig. 1B). Synthetic cannabinoids, such as WIN 55,212-2, CP 55,940, and Δ^9-THC, a major psychoactive component of cannabis, at the concentration of 10 μM, did not alter the amount of $^{45}Ca^{2+}$ in the vesicles in either control or flux conditions (Fig. 1C).

3.2. Effects of endogenous and synthetic cannabinoids on the binding of [^{3}H]PN 200–110

In T-tubule membrane preparations, the high affinity binding of the dihydropyridine (DHP)-class of Ca^{2+} channel ligands has been characterized previously (Fosset et al., 1983; Flockerzi et al., 1986; Dunn, 1989; Dunn et al., 1994). Equilibrium curves for the binding of [^{3}H]PN 200–110, in the presence and absence of the 2-arachidonoylglycerol and R-methanandamide, are presented in Fig. 2A. Both 2-arachidonoylglycerol and R-methanandamide (10 μM) caused a significant inhibition of the specific binding of [^{3}H]PN 200–110. Maximum binding activities (B_{max}) of [^{3}H]PN 200–110 were 28.6, 13.4, and 15.2 pmol/mg for controls, 2-arachidonoylglycerol and R-methanandamide, respectively. The apparent affinity (K_d) of the receptor for [^{3}H]PN 200–110 were 248, 259, and 263 pM for controls, 2-arachidonoylglycerol, and R-methanandamide, respectively.

However, WIN 55,212-2, CP 55,940, and Δ^9-THC did not alter specific binding of [^{3}H]PN 200–110 in the concentration range of 0.1 to 100 μM (Fig. 2B). 2-Arachidonoylglycerol and R-methanandamide, WIN 55,212-2, CP 55,940, and Δ^9-THC were also investigated comparatively for their effects on the displacement of specific binding of [^{3}H]PN 200–110 from rabbit T-tubule membranes (Fig. 2C). Both 2-arachidonoylglycerol and R-methanandamide (30–100 μM) inhibited the specific binding of [^{3}H]PN 200–110 completely. The IC_{50} values for 2-arachidonoylglycerol and R-methanandamide were 3.2+0.4

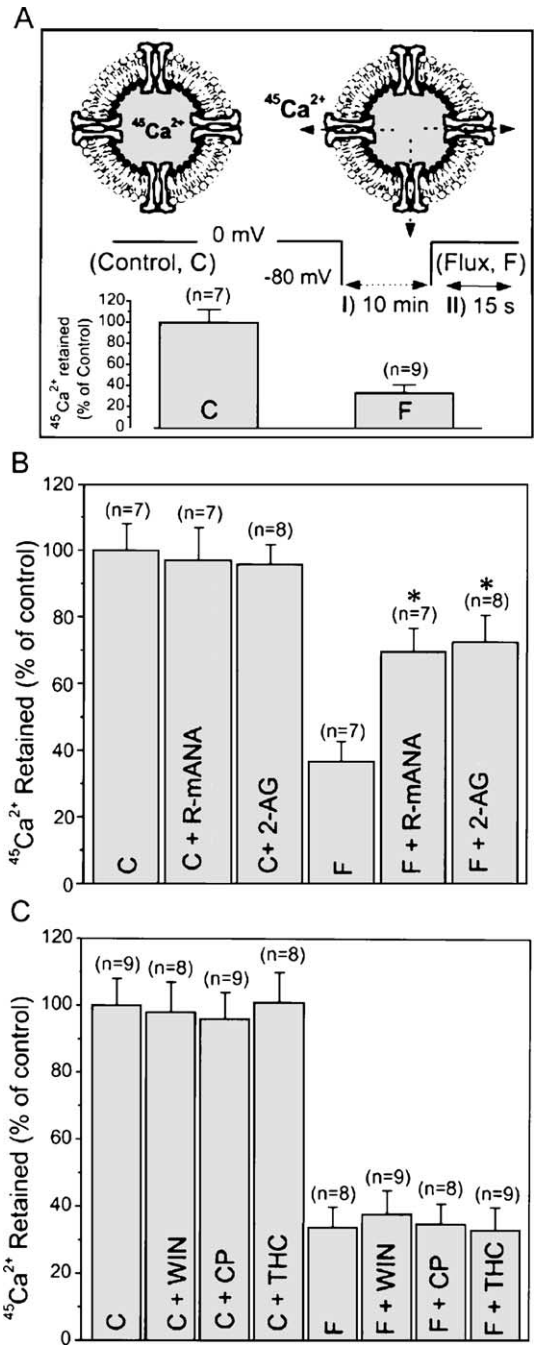

Fig. 1. The effects of endocannabinoids and synthetic cannabinoids on $^{45}Ca^{2+}$ efflux through T-tubule membranes. (A) Schematic presentation of isolated, inside–out T-tubule membranes. Two steps of the protocol used to induce $^{45}Ca^{2+}$ fluxes are denoted by I and II, respectively. Inset shows the amounts of $^{45}Ca^{2+}$ in vesicles measured before (C) and after (F) depolarizations were presented as percentage bars. (B) Effects of endocannabinoids on depolarization-induced $^{45}Ca^{2+}$ effluxes. (C) Effects of synthetic cannabinoids on depolarization-induced $^{45}Ca^{2+}$ effluxes. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represent the S.E.M. * indicates statistical significance at the level of $P<0.05$. All compounds tested at the concentration of 10 μM. C, control conditions; F, efflux conditions; ANA, anandamide; R-mANA, R-methanandamide; 2-AG, 2-arachidonoylglycerol; CP, CP 55,940; WIN, WIN 55,212-2; THC, Δ^9-tetrahydocannabinol.

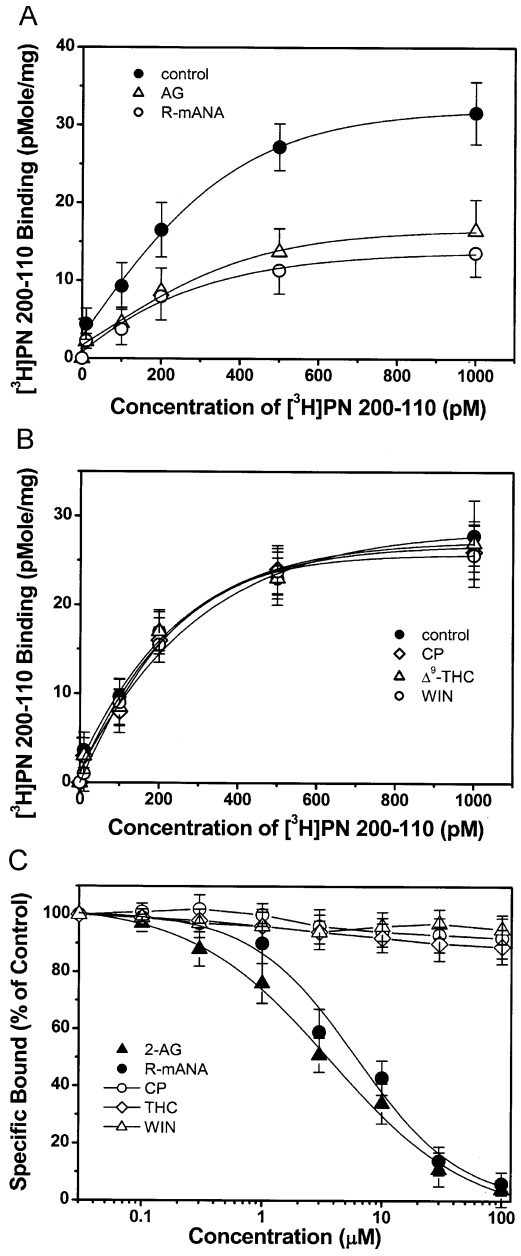

Fig. 2. The effects of endocannabinoids and synthetic cannabinoids on the specific binding of [^{3}H]PN 200–110 to T-tubule membranes. (A) Specific binding as a function of the concentration of ^{3}H]PN 200–110 in controls, in the presence of 2-arachidoylglycerol and R-methanandamide. (B) Specific binding as a function of the concentration of [^{3}H]PN 200–110 in controls, in the presence of CP, CP 55,940; WIN, WIN 55,212-2; THC, Δ^9-tetrahydocannabinol. Data are presented as the arithmetic means of four to five experimental measurements. Solid lines were calculated from the best-fit parameters obtained by nonlinear curve fitting to a single-site binding equation. (C) The effects of increasing concentrations of R-methanandamide (R-mANA), 2-arachidonoylglycerol (2-AG), CP 55,940 (CP), WIN 55,212-2 (WIN), and Δ^9-tetrahydocannabinol (THC) on the displacement of specific binding of [^{3}H]PN 200–110 to T-tubule membranes. Data are expressed as percentage of control. The IC_{50} values were obtained from nonlinear regression fits of the data points. Membranes were incubated with 0.5 nM [^{3}H]PN 200–110 for 1 h in the presence of increasing concentrations of test compound in the medium. Bound and free [^{3}H]PN 200–110 were separated by filtration. Symbols are the means of four to five experiments. Vertical lines on each data point represent the S.E.M.

and 7.1+2.3 μM, respectively, with corresponding slope factors of 0.7 and 0.9.

3.3. Studies to determine the effects of metabolic products of endocannabinoids

The above results from flux and radioligand-binding experiments suggested that the interaction with VDCCs is endocannabinoid-specific. However, the endocannabinoids tested in these experiments have metabolic products: arachidonic acid and ethanolamine are contained in anandamide, whereas glycerol and arachidonic acid are found in 2-arachidonoylglycerol. Therefore, we attempted to determine whether these metabolic products mediate endocannabinoid inhibition of depolarization-induced Ca^{2+} flux responses. Incubation of T-tubule membrane preparations with 10 μM of ethanolamine or glycerol for 10 min did not alter the amount of $^{45}Ca^{2+}$ in the vesicles in either control (data not shown, n=6–9) or in flux conditions (Fig. 3A), whereas within the same batch of membrane preparations, 10 μM of R-methanandamide or arachidonic acid inhibited the flux responses significantly without affecting the content of $^{45}Ca^{2+}$ in the vesicles under control conditions (P<0.05, ANOVA, n=7–8, Fig. 3A).

In radioligand-binding experiments, the effects of ethanolamine, glycerol, arachidonic acid, and R-methanandamide were investigated comparatively on the specific binding of DHP-class ligand [^{3}H]PN 200–110. Equilibrium curves for the binding of [^{3}H]PN 200–110 in controls and in the presence of ethanolamine, glycerol, arachidonic acid and R-methanandamide are presented in Fig. 3B.

At a concentration of 10 μM, R-methanandamide and arachidonic acid caused a significant inhibition of the specific binding of [^{3}H]PN 200–110. Maximum binding activities (B_{max}) of [^{3}H]PN 200–110 were 28.7, 14.8, and 12.9 pmol/mg for controls, and in the presence of arachidonic acid, and R-methanandamide, respectively. The apparent affinity (K_d) of the receptor for [^{3}H]PN 200–110 was 216, 221, and 227 pM for controls, and in the presence of arachidonic acid, and R-methanandamide, respectively. At a concentration of 10 μM, ethanolamine or glycerol, on the other hand, did not cause an appreciable change on the characteristics of the [^{3}H]PN 200–110 binding (Fig. 3B). Values for B_{max} were 28.7, 29.4, and 27.3 pmol/mg for controls, ethanolamine and glycerol, respectively. The K_d values were 198, 204 and 213 pM for controls, ethanolamine and glycerol, respectively.

R-methanandamide, arachidonic acid, ethanolamine and glycerol were also investigated comparatively for their effects on the displacement of specific [^{3}H]PN 200–110 binding from T-tubule membranes (Fig. 3C). In the concentration range used (0.1 to 100 μM), R-methanandamide and arachidonic acid caused a significant inhibition on the specific binding of [^{3}H]PN 200–110 (Fig. 3C). The values for IC_{50} and slope factor for R-methanandamide and arachidonic acid were 2.9±0.3 μM and 0.8, and 8.7±0.4

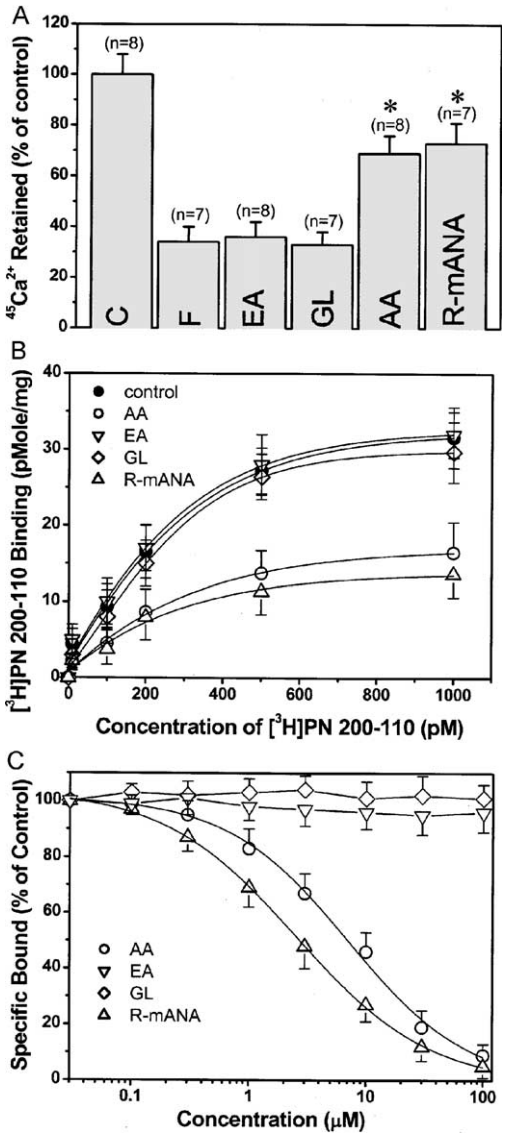

Fig. 3. Effects of different chemical moieties of endocannabinoids on depolarization-induced $^{45}Ca^{2+}$ effluxes through T-tubule membranes and specific binding of [^{3}H]PN 200–110. (A) Effects of R-methanandamide, arachidonic acid, ethanolamine, or glycerol on depolarization-induced $^{45}Ca^{2+}$ effluxes. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represent the S.E.M. * indicates statistical significance at the level of P<0.05. All compounds tested at the concentration of 10 μM. C, control conditions; F, efflux conditions; R-mANA, R-methanandamide; AA, arachidonic acid; EA, ethanolamine; GL, glycerol. (B) Effects of R-methanandamide, arachidonic acid, ethanolamine, or glycerol on specific binding as a function of the concentration of [^{3}H]PN 200–110. Data are presented as the arithmetic means of four experimental measurements in the absence and presence 10 μM of R-methanandamide, arachidonic acid, ethanolamine, or glycerol. The incubation time was 60 min at 23 °C, pH 7.5. Equivalent samples were incubated with 5 μM of unlabeled nifedipine in order to determine nonspecific binding. (C) The effects of increasing concentrations of R-methanandamide, ethanolamine, glycerol, and arachidonic acid on the displacement of specific binding of [^{3}H]PN 200–110 to T-tubule membranes. The data are expressed as percentage of control. T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200–110 for 1 h in the presence of increasing concentrations of compounds tested in the medium. Bound and free [^{3}H]PN 200–110 were separated by filtration. Symbols are the means of at least five experiments. Vertical lines represent S.E.M.

μM and 0.9, respectively. Ethanolamine or glycerol in the concentration range tested (0.1 to 100 μM) did not cause an appreciable effect on the specific binding of [^{3}H]PN 200–110 (Fig. 3C).

3.4. Studies to determine the structure of fatty acids mediating the effects of endocannabinoids

Similar to endocannabinoids, alcohols and general anesthetics are lipophilic agents, and they also inhibit VDCCs of smooth (Hawtorn et al., 1992), cardiac (Habuchi et al., 1995), and skeletal muscles (Oz et al., 2001, 2002b,c). It has been proposed that a hydrophobic binding site or a hydrophobic pocket mediates the effects of alcohols on various ion channels (Franks and Lieb, 1994; Peoples et al., 1996). Alcohols with different carbon chain lengths have been widely used to investigate the physical nature of this hydrophobic site (Franks and Lieb, 1994; Peoples et al., 1996; Oz et al., 2001, 2002c). We took a similar approach and employed the following fatty acids with two double bonds at the concentration of 10 μM, hexadecadienoic acid (16:2), octadecadienoic acid (18:2), and eicosadinoic acid (20:2) and docosadienoic acid (22:2) to investigate if the effects of fatty acids are altered by changing their carbon chain length. Incubation with 10 μM of hexadecadienoic acid for 10 min did not cause significant alterations in the amount of $^{45}Ca^{2+}$ in T-tubule vesicles either in control (data not shown; n=6–7) or in flux conditions. However, 10 μM of octadecadienoic acid, eicosadinoic acid, and docosadienoic acid caused significant inhibition of depolarization-induced $^{45}Ca^{2+}$ effluxes (Fig. 4A) with no alteration in the amount of $^{45}Ca^{2+}$ in T-tubule vesicles in either control or flux conditions (data not shown; n=5–7).

In earlier studies, it was found that changing the number of double bonds in fatty acids alters their effects on VDCCs on smooth (Shimada and Somlyo, 1992) and cardiac muscle (Xiao et al., 1997) and hippocampal neurons (Vreugdenhil et al., 1996) significantly. For this reason, we have tested the effect of octadecanoic (18:0; stearic acid), octadecenoic (18:1; oleic), octadecadienoic (18:2; linoleic), and octadecatrienoic (18:3; linolenic) acids at the concentration of 10 μM. Stearic acid and oleic acid did not change the amount of $^{45}Ca^{2+}$ in T-tubule vesicles either in control or in flux conditions. On the other hand, both linoleic and linolenic acids caused significant inhibition of depolarization-induced $^{45}Ca^{2+}$ effluxes (Fig. 4B). Similarly, although 10 μM of eicosanoic acid (20:0; arachidic) did not alter $^{45}Ca^{2+}$ fluxes, eicosatetraenoic (20:4; arachidonic acid) and eicosapentaenoic acid (20:5) inhibited the depolarization-induced $^{45}Ca^{2+}$ fluxes significantly (data not shown; n=6–9). Eicosatetraynoic acid, the acetylenic analogue of arachidonic acid, is identical to arachidonic acid except that the four double bonds of arachidonic acid are changed to triple bonds. We have tested the effect of eicosatetraynoic acid to probe if the double-bond structure of arachidonic acid plays a functional role in inhibiting depolarization-induced $^{45}Ca^{2+}$ fluxes.

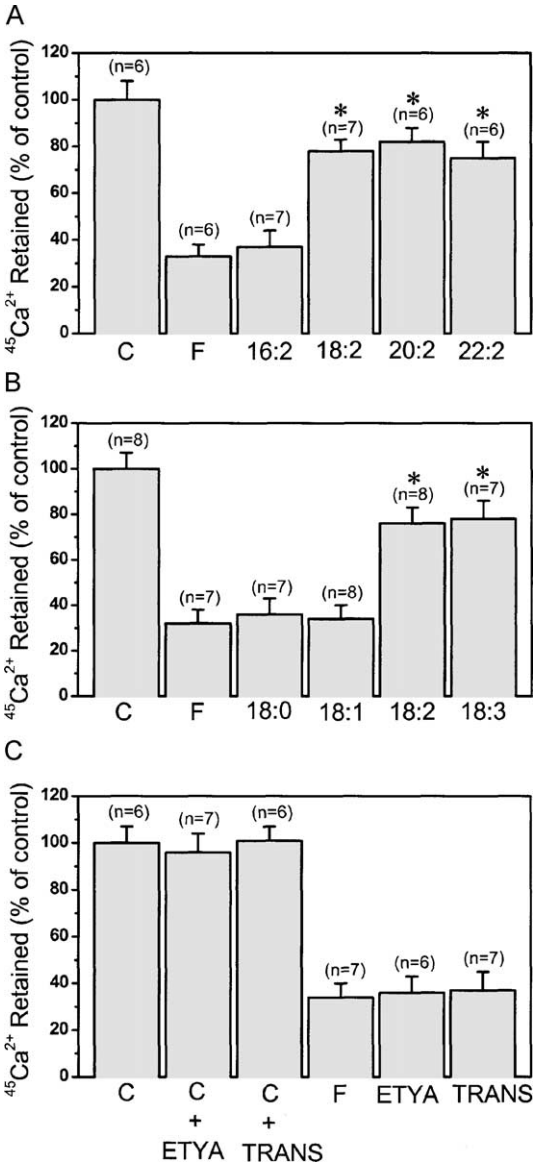

Fig. 4. Effects of fatty acids with different length of acyl chains and different numbers of double bonds on depolarization-induced $^{45}Ca^{2+}$ effluxes through T-tubule membranes. (A) Effects of 10 μM, hexadecadienoic acid (16:2), octadecadienoic acid (18:2), and eicosadinoic acid (20:2) and docosadienoic acid (22:2) on $^{45}Ca^{2+}$ flux responses. (B) Effects of increasing the number of double bonds in octadecanoic (18:0) acid on $^{45}Ca^{2+}$ flux responses. (C) Effects of eicosatetraynoic acid and linoelaidic acid on depolarization-induced $^{45}Ca^{2+}$ effluxes. The numbers of experiments (n) are presented on top of each column. Vertical lines on top of the columns represent the S.E.M. * indicates level of significance at P<0.05. C, control conditions; F, efflux conditions; N:N, number of carbon atoms:number of double bonds in fatty acid. Systematic names of fatty acids are given in the text.

Incubation with 10 μM eicosatetraynoic acid for 10 min did not cause significant alterations in the amount of $^{45}Ca^{2+}$ in T-tubule vesicles in either control or flux conditions (Fig. 4C; P>0.05, ANOVA, n=7–8).

Unsaturated fatty acids can exist in either the *cis*- or *trans*-form depending on the configuration of the hydrogen atoms attached to the carbon atoms joined by the double

bonds. Several earlier studies indicate that biological effects of polyunsaturated fatty acids with the same chain length and number of double bonds can vary significantly depending on *cis*- or *trans*-configuration of double bonds in their structure (Xiao et al., 1997; Vreugdenhil et al., 1996). For this reason, we have tested the effects of all *trans*-double bond isomer of linoleic acid, known as linoelaidic acid. Incubation with 10 μM linoelaidic acid for 10 min did not cause significant alterations in the amount of $^{45}Ca^{2+}$ in T-tubule vesicles neither in either control or in flux conditions (Fig. 4C; $P>0.05$, ANOVA, $n=6–8$).

Under our depolarizing conditions, a considerable amount of $^{45}Ca^{2+}$ remains in the vesicle (30–35% of total $^{45}Ca^{2+}$, Fig. 1). Since fatty acids have been reported to bind Ca^{2+} with an efficiency that varies with the chain length of the fatty acids (Leaf et al., 2002), we have used A23187 Ca^{2+} ionophore (2 μM) to assess the total releasable $^{45}Ca^{2+}$ in the presence of some of the fatty acids used in this study. In the presence of A23187, the amount of $^{45}Ca^{2+}$ decreased to 5–10% of total $^{45}Ca^{2+}$ in vesicles. There were no significant differences between the amounts of retained of $^{45}Ca^{2+}$ in T-tubule vesicles in the presence of A23187 alone or A23187 and 10 μM of hexadecadienoic ($n=6$), octadecadienoic ($n=5$), eicosadienoic ($n=5$), octadecenoic ($n=5$), linoelaidic ($n=5$), and eicosatetraenoic acid ($n=6$), suggesting that fatty acids do not bind a significant amount of $^{45}Ca^{2+}$ in vesicles (data not shown).

In radioligand-binding experiments, the effects of a series of fatty acids with an increasing number of double bonds were investigated on the specific binding of DHP-class ligand [^{3}H]PN 200–110. Equilibrium curves for the binding of [^{3}H]PN 200–110 in controls and in the presence of saturated fatty acid, octadecanoic acid (18:0; stearic acid), monounsaturated fatty acid, octadecenoic acid (18:1; oleic), polyunsaturated fatty acids octadecadienoic (18:2; linoleic), and octadecatrienoic (18:3; linolenic) acids are presented in Fig. 5A ($n=4–5$). In the presence of stearic and oleic acids (10 μM), there were no significant changes on the maximum binding activities (B_{max}) or on the apparent affinity (K_d) of the receptor for [^{3}H]PN 200–110. On the other hand, linoleic and linolenic acids (10 μM) caused significant inhibition of the specific binding of the [^{3}H]PN 200–110 binding (Fig. 5A). Values for B_{max} were 18.7, 17.2, and 17.9 pmol/mg for controls, linoleic and linolenic acids, respectively. The K_d values were 197, 201, and 203 pM for controls, linoleic, and linolenic acids, respectively. These fatty acids were also investigated comparatively for their effects on the displacement of specific [^{3}H]PN 200–110 binding from T-tubule membranes (Fig. 5B; $n=4–5$). In the concentration range used (0.1 to 100 μM), polyunsaturated fatty acids, including linoleic (18:2) and linolenic (18:3) acids, caused a significant inhibition on the specific binding of [^{3}H]PN 200–110. The values for IC_{50} and slope factor for linoleic and linolenic acids were 12.1 μM and 1.1, and 16.4 μM and 1.1, respectively. Stearic and oleic acids in the concentration range tested (0.1 to 100 μM) did not cause an appreciable effect on the specific binding of [^{3}H]PN 200–110 (Fig. 5B).

The effects of eicosatetraynoic acid and linoelaidic acid were investigated on the specific binding of DHP-class ligand [^{3}H]PN 200–110. Equilibrium curves for the binding of [^{3}H]PN 200–110 in controls and in the presence of eicosatetraynoic acid and linoelaidic acid are presented in Fig. 5C ($n=4–5$). At a concentration of 10 μM, eicosatetraynoic acid or linoelaidic acid did not cause a significant change on the specific binding of [^{3}H]PN 200–110. Values for B_{max} were 25.7, 27.1, and 24.3 pmol/mg for controls, eicosatetraynoic acid, and linoelaidic acid, respectively. The K_d values were 228, 219, and 231 pM for controls, eicosatetraynoic acid, and linoelaidic acid, respectively. These fatty acids were also investigated for their effects on the displacement of specific [^{3}H]PN 200–110 binding from T-tubule membranes (Fig. 5D; $n=4–5$). In the concentration range used (0.1 to 100 μM), eicosatetraynoic acid or linoelaidic acid did not cause an appreciable effect on the specific binding of [^{3}H]PN 200–110.

4. Discussion

The main finding of this study is that endocannabinoids, but not synthetic cannabinoids, inhibit the function of VDCCs in T-tubule membranes. This inhibition is independent of cannabinoid receptor activation and mediated by fatty acid moieties of the endocannabinoids.

In our earlier studies, we have shown that inhibition of Ca^{2+} fluxes by anandamide was not altered by the presence of phenylmethylsulfonyl fluoride (PMSF, 0.2 mM), amidohydrolase inhibitor, indomethacin (5 μM), cyclooxygenase inhibitor, and superoxide dismutase (SOD, 50 U/ml), antioxidant enzyme (Oz et al., 2000). In correlation with these findings, R-methanandamide, a metabolically stable chiral analog of anandamide that is more resistant than anandamide to hydrolytic inactivation by fatty acid amide hydrolase (Abadji et al., 1994), was equally effective as anandamide or 2-arachidonoylglycerol (Fig. 1). Furthermore, ethanolamine and glycerol, metabolic products of anandamide and 2-arachidonoylglycerol, were ineffective in inhibiting depolarization-induced Ca^{2+} fluxes (Fig. 2). Based on these results, it is unlikely that the observed effects of endocannabinoids are mediated through their metabolically active products.

In earlier studies using a variety of cell types, it was reported that both anandamide and 2-arachidonoylglycerol cause the release of Ca^{2+} from intracellular stores and thus increase the concentration of intracellular Ca^{2+} (for a review, Howlett and Mukhopadhyay, 2000). Since VDCCs have been shown previously to be modulated by changes in intracellular Ca^{2+} concentrations (Feldmeyer et al., 1993; Soldatov et al., 1998; Oz et al., 1998), the absence of intracellular organelles and inside–out orientation of T-tubule vesicles (Fosset et al., 1983; Flockerzi et al., 1986;

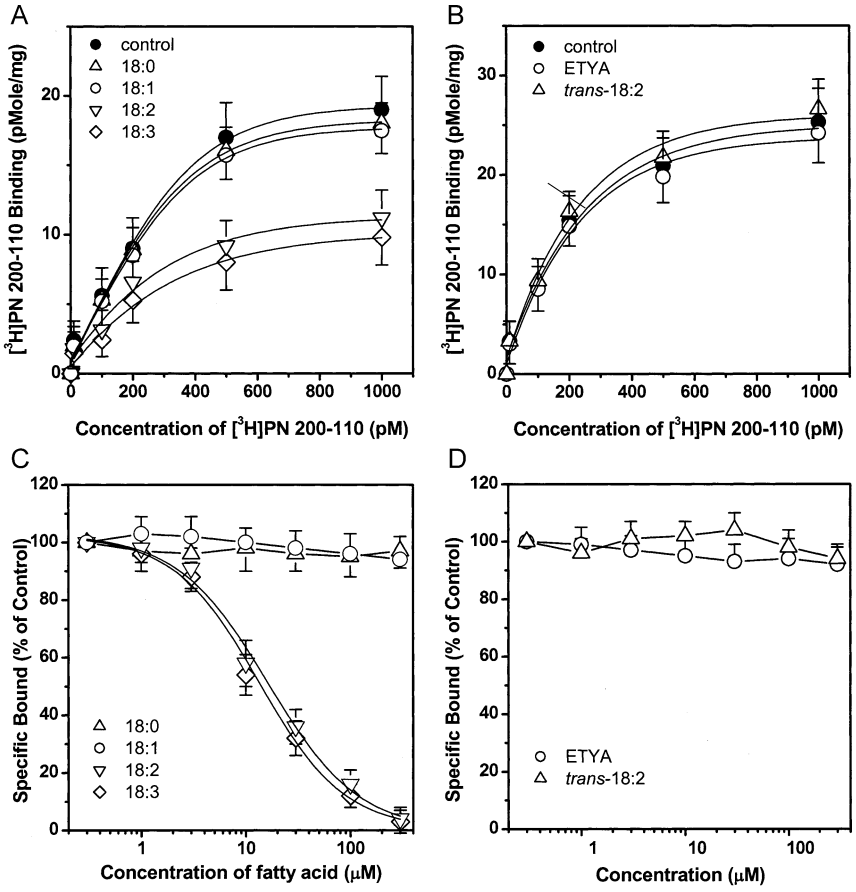

Fig. 5. Effects of fatty acids with different numbers of double bonds, eicosatetraynoic acid and linoelaidic acid on the specific binding of [^{3}H]PN 200–110 to T-tubule membranes, (A) Specific binding as a function of the concentration of [^{3}H]PN 200–110. Data are presented as the arithmetic means of five experimental measurements in the absence (control) and presence (10 μM) of octadecanoic (18:0), octadecenoic acid (18:1), octadecadienoic (18:2), and octadecatrienoic (18:3) acids. The incubation time was 60 min at 23 °C, pH 7.5. Equivalent samples were incubated with 5 μM of unlabeled nifedipine in order to determine nonspecific binding. (B) The decrease of specific binding of [^{3}H]PN 200–110 to T-tubule membranes by increasing concentrations of fatty acids. The data are expressed as percentage of control. T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200–110 for 1 h in the presence of increasing concentrations of fatty acids. Bound and free [^{3}H]PN 200–110 were separated by filtration. Symbols are the means of at least five experiments. Vertical lines represent S.E.M. (C) Specific binding as a function of the concentration of [^{3}H]PN 200–110. Data are presented as the arithmetic means of five experimental measurements in the absence and presence (10 μM) of eicosatetraynoic acid and linoelaidic acid. The incubation time was 60 min at 23 °C, pH 7.5. Equivalent samples were incubated with 5 μM of unlabeled nifedipine in order to determine nonspecific binding. (D) The decrease of specific binding of [^{3}H]PN 200–110 to T-tubule membranes by increasing concentrations of eicosatetraynoic acid or linoelaidic acids. The data are expressed as percentage of control. T-tubule membranes were incubated with 0.5 nM [^{3}H]PN 200–110 for 1 h in the presence of increasing concentrations of fatty acids. Bound and free [^{3}H]PN 200–110 were separated by filtration. Symbols are the means of at least five experiments. Vertical lines represent S.E.M.

Rosemblatt and Scales, 1989; Dunn, 1989) makes it unlikely that the observed inhibitory effects of endocannabinoids involves changes in intracellular Ca^{2+} levels. Also, since these membranes are devoid of any plasmalemmal Ca^{2+} pump activity (Rosemblatt and Scales, 1989; Dunn, 1989), the reported effects of cannabinoids on Ca^{2+}-ATPase activity (Collins and Haavik, 1979; Dalterio et al., 1987) are unlikely to interfere with $^{45}Ca^{2+}$ fluxes measured in the present study. Furthermore, since changing the lipid composition of bilayer membranes has been shown to affect the functional properties of VDCCs significantly (Glossmann and Ferry, 1983; Coronado, 1987), it is advantageous to employ T-tubule vesicles in which the native membranes' composition is preserved. Thus, T-tubule membrane preparations provide a model system in which the effects of cannabinoids and fatty acids on both radioligand binding and functional aspects of VDCCs can be studied in a relatively isolated assay system.

In previous studies, we have demonstrated that DHP-class calcium channel antagonists inhibit depolarization-induced $^{45}Ca^{2+}$ effluxes in a stereo-specific manner (Oz et al., 1992, 1993) and anandamide interacts functionally with the effects of the DHP-class antagonists and agonists on $^{45}Ca^{2+}$ effluxes, indicating that flux responses inhibited by endocannabinoids are mediated by L-type VDCCs in T-tubule membranes (Oz et al., 2000). It is important to note that depolarizations induced by the changes in extravesicular concentrations of K^+ have been recorded and by using voltage-sensitive fluorescent dyes and depolarizations have been shown to be consistent with values predicted by the Nernst equation (Dunn, 1989).

Although the potential is assumed to be fixed by "valinomycin" clamping at 0 mV during depolarization phase, considering a high concentration gradient for Ca^{2+} ions (5 mM inside vs. 100 nM outside), the contribution of Ca^{2+} to membrane potential cannot be neglected around 0 mV, the steepest region of the voltage dependency of activation curve for skeletal type VDCCs (Morrill and Cannon, 1999). Since the maximal conductance of the channels are reached at potential higher than 0 mV (Morrill and Cannon, 1999), small deviations from the intended voltage could cause considerable changes in the amount of $^{45}Ca^{2+}$ passed through VDCCs. For this reason, we have conducted some experiments with low–high K^+ concentrations (40 mM KCl outside). This partial depolarizing condition has been used to mimic the effect of extracellular K^+ accumulation during maintained tetanic contractions and has also been shown to induce significant $^{45}Ca^{2+}$ fluxes through VDCCs (Oz and Frank, 1991; Oz et al., 1992, 1993). Results of our experiments conducted under partial depolarizations indicated that both anandamide and 2-arachidonoylglycerol inhibited $^{45}Ca^{2+}$ fluxes, significantly (n=5–7, data not shown) suggesting that under different conductance levels, VDCCs could be inhibited by endocannabinoids.

In our previous studies, it was found that the inhibition by anandamide of Ca^{2+} fluxes through T-tubule vesicles is not sensitive to pertussis toxin treatments, and not altered by cannabinoid CB_1 receptor antagonist SR141716A (Oz et al., 2000) or CB_2 receptor antagonist SR144528 (our unpublished results). In addition, RNA expression of cannabinoid CB_1 or CB_2 receptors has not been detected in mammalian skeletal muscle (Howlett et al., 2002). These findings indicate that the effects of endocannabinoids on VDCCs are not mediated by the activation of cannabinoid receptors. In recent studies, endocannabinoids were shown to inhibit voltage-dependent Na^+, K^+, and Ca^{2+} channels (Poling et al., 1996; Oz et al., 2000; Chemin et al., 2001; Maingret et al., 2001; Nicholson et al., 2003; Guo and Ikeda, 2004), ligand-gated channels, such as 5-HT_3 receptors (Barann et al., 2002; Oz et al., 2002a) and nicotinic acetylcholine receptors (Oz et al., 2003, 2004a), and that these effects were also found to be insensitive to SR141716A and/or to treatments with pertussis toxin. Interestingly, in a recent study, it was found that both anandamide and arachidonic acid cause a functional conversion between A-type and delayed rectifier K^+ channels (Oliver et al., 2004; Hilgemann, 2004). In this study (Oliver et al., 2004), a delayed rectifier K^+ channel was converted to rapidly desensitizing A-type K^+ channel by application of anandamide in oocyte expression system that does not express endogenous cannabinoid receptors (Henry and Chavkin, 1995).

In the present study, functional and radioligand-binding experiments indicate that arachidonic acid, a fatty acid metabolite of the endocannabinoids, interacts with VDCCs. On the other hand, other endocannabinoid metabolites, such as ethanolamine and glycerol, were found to have no significant effect on either Ca^{2+} fluxes or on the specific binding of [^{3}H]PN 200–110. Further studies indicated that interaction of fatty acids with VDCCs is not specific to arachidonic acid, but most of the long-chain fatty acids (C18–C22) containing at least two double bonds also have inhibitory effects on VDCCs. These findings are in agreement with earlier studies indicating that at the concentrations used in this study, arachidonic acid and other polyunsaturated long-chain fatty acids, but not saturated fatty acids, inhibit Ca^{2+} currents mediated through L-type VDCC in both smooth and cardiac muscle preparations (Shimada and Somlyo, 1992; Xiao et al., 1997; Mamas and Terrar, 2001; Leaf et al., 2002). These studies also report that the number of double bonds in fatty acids significantly increases the potency of their inhibitory effects on VDCCs of ventricular myocytes (Xiao et al., 1997) and that of hippocampal neurons (Vreugdenhil et al., 1996). In our studies, the IC_{50} values on radioligand-binding experiments were affected significantly by the chemical structure of the fatty acids. However, concentration dependency of their effects in functional assays could not be studied, since our attempts to obtain gradual $^{45}Ca^{2+}$ effluxes by decreasing depolarization times to 4 s and/or using partial depolarization conditions were not successful. In the concentration range of 1–10 μM used in depolarization-induced $^{45}Ca^{2+}$ effluxes, endocannabinoids have been reported to activate vanilloid receptor-mediated ion currents (Benham et al., 2002) or inhibit voltage-dependent Na^+ channels (Nicholson et al., 2003) and gap junctions (Venance et al., 1995).

Eicosatetraynoic acid, identical to arachidonic acid except that the four double bonds of arachidonic acid are changed to four triple bonds, was completely ineffective in inhibiting depolarization-induced $^{45}Ca^{2+}$ effluxes. These results indicate that the presence of double bonds is essential in mediating the effect of arachidonic acid on VDCCs. Eicosatetraynoic acid has the same effect on membrane fluidity as other fatty acids used in this study (Meves, 1994); thus, the absence of any effect of eicosatetraynoic acid shows that nonspecific changes on the physical properties of the lipid bilayer do not underlie the effect of fatty acids on depolarization-induced of $^{45}Ca^{2+}$ effluxes in T-tubule membranes.

The ability of endocannabinoids to inhibit the specific binding of the radioligand [^{3}H]PN 200–110 to T-tubule membranes suggests that they interact with DHP binding sites in the VDCC. In earlier studies in cortical, cardiac (Johnson et al., 1993), and skeletal muscle (Oz et al., 2000; Shiamsue et al., 1996) membranes, no effects of PMSF on anandamide inhibition of DHP binding were found, indicating that arachidonic acid produced by the hydrolysis of anandamide is not involved in this effect. However, the results of both functional and radioligand-binding studies indicate that fatty acid moieties of the endocannabinoids, such as arachidonic acid, are important in mediating their interaction with VDCCs. In the concentration range used in this study, arachidonic acid was reported to inhibit the specific binding of DHPs in skeletal (Shiamsue et al., 1996;

Oz et al., 2000) and cardiac muscle (Xiao et al., 1997; Leaf et al., 2002). In earlier studies, we have reported that DHP-class antagonists inhibit depolarization-induced $^{45}Ca^{2+}$ effluxes in a stereo-specific manner at low micromolar concentrations (Oz et al., 1992, 1993) and anandamide interact functionally with the effect of DHPs on VDCCs (Oz et al., 2000). The presence of a low-affinity binding site for DHPs on VDCCs has been demonstrated in T-tubule membranes (Dunn and Bladen, 1991, 1992).

Allosteric interaction of these compounds with this low-affinity binding site may mediate their functional effects on VDCCs. In earlier studies, it was shown that the binding of DHPs and the function of VDCCs were affected by phospholipids and fatty acids added to assay medium (Glossmann and Ferry, 1983; Coronado, 1987). For the other lipophilic agents including alcohols and general anesthetics, recent findings suggest strongly that they exert their modulatory effects by acting directly on the ion channels rather than disturbing the structure of lipid-bilayer membranes (Franks and Lieb, 1994; Peoples et al., 1996). Important evidence supporting this hypothesis is the loss of the biological effects of alcohols when their carbon chains exceed a certain length. This property was observed on both ligand- and voltage-gated ion channels (Franks and Lieb, 1994; Peoples et al., 1996; Oz et al., 2002c) and it is known as the "cut-off" phenomenon. Similarly, in this and earlier studies (Shimada and Somlyo, 1992), it has been found that the interaction of fatty acids with VDCCs are limited to those with long carbon chain lengths (C18 to C22 in this study) containing a minimum of two double bonds. It is likely that a hydrophobic binding site(s) for fatty acids is in the inner part of the lipid membrane and only the fatty acids having at least two or more double bonds with *cis*-configuration interact with this site(s). However, results of radioligand-binding experiments indicate that K_d values for anandamide, R-methanandamide, and 2-arachidonoylglycerol (Oz et al., 2000 and Fig. 2C) are approximately three to four times lower than those for arachidonic acid (20:4, Oz et al., 2000), linoleic acid (18:2, Fig. 5B), and linolenic acid (18:3, Fig. 5B), suggesting that polar head groups are also important for the effects of endocannabinoids tested in this study.

Recently, O.S. Anderson and colleagues, working mainly on gramicidin channels, hypothesized that if the hydrophobic length of the transmembrane domains of the ion channels does not match the hydrophobic thickness of the membrane phospholipid bilayer, such a mismatch would create stress between the channel and membrane (Lundbaek et al., 1996; Andersen et al., 1999). Frequently, the hydrophobic lengths of the ion channels are slightly less than the hydrophobic thickness of the acyl chains of the phospholipid fatty acids. As a result, this tension requires that the thickness of the cell membrane be decreased at its contact with the interfaces of the transmembrane regions of the ion channel so that their hydrophobic regions match and affect the conformational state and conductance of the ion channel. Anderson and colleagues also reason that the tension on the ion channels would be altered if the agents that incorporate into the interface between the phospholipid membrane and ion channel reduce the curvature and the tension on the channel protein. They have reported that nonionic detergent, such as Triton X-100 which forms micelles but bears no chemical similarity to fatty acids, also affects gramicidin channel and voltage-sensitive Na^+ and Ca^{2+} channels (Lundbaek et al., 1996; Andersen et al., 1999). In line with their hypothesis, we have found that Triton X-100 inhibits depolarization-induced $^{45}Ca^{2+}$ effluxes in T-tubule membranes (10 μM, n=5, our unpublished results). Interestingly, we have recently shown that Triton X-100 can inhibit nicotinic ACh receptors (Oz et al., 2004b), which are also target proteins for direct actions of endocannabinoids. Although we cannot differentiate between two hypotheses, i.e., acting through a hydrophobic binding site located on the protein or by altering the membrane compression on the channel, our results indicate that endocannabinoids, but not synthetic cannabinoids, inhibit depolarization-induced $^{45}Ca^{2+}$ effluxes in a cannabinoid receptor-independent manner, and their interaction with VDCCs are mediated by fatty acid moieties of endocannabinoids.

Acknowledgments

The authors thank Mrs. Mary Pfeiffer and Dr. Alex Hoffman of NIDA/NIH for their helpful reading of the manuscript. Drs. Y.T and M.D. greatly appreciate generous financial support by Mr. Ugur Tuncay of Tuncay Inc., Ankara, Turkey, during their studies.

References

Abadji, V., Lin, S., Taha, G., Griffin, G., Stevenson, L.A., Pertwee, R.G., Makriyannis, A., 1994. (*R*)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. J. Med. Chem. 37, 1889–1893.

Andersen, O.S., Nielsen, C., Maer, A.M., Lundbaek, J.A., Goulian, M., Koeppe II, R.E., 1999. Ion channels as tools to monitor lipid bilayer–membrane protein interactions: gramicidin channels as molecular force transducers. Methods Enzymol. 294, 208–224.

Baker, D., Pryce, G., 2003. The therapeutic potential of cannabis in multiple sclerosis. Expert Opin. Investig. Drugs 12, 561–567.

Barann, M., Molderings, G., Bruss, M., Bonisch, H., Urban, B.W., Gothert, M., 2002. Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. Br. J. Pharmacol. 137, 589–596.

Benham, C.D., Davis, J.B., Randall, A.D., 2002. Vanilloid and TRP channels: a family of lipid-gated cation channels. Neuropharmacology 42, 873–888.

Chemin, J., Monteil, A., Perez-Reyes, E., Nargeot, J., Lory, P., 2001. Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. EMBO J. 20, 7033–7040.

Collins, F.G., Haavik, C.O., 1979. Effects of cannabinoids on cardiac microsomal CaATPase activity and calcium uptake. Biochem. Pharmacol. 28, 2303–2306.

Coronado, R., 1987. Planer bilayer reconstitution of calcium channels: lipid effects on single-channel kinetics. Circ. Res. 61, I46–I52.

Dalterio, S.L., Bernard, S.A., Esquivel, C.R., 1987. Acute delta 9-tetrahydrocannabinol exposure alters Ca^{2+} ATPase activity in neuroendocrine and gonadal tissues in mice. Eur. J. Pharmacol. 137, 91–100.

Dunn, S.M.J., 1989. Voltage-dependent calcium channels in skeletal muscle transverse tubules: measurements of calcium efflux in membrane vesicles. J. Biol. Chem. 264, 11053–11060.

Dunn, S.M.J., Bladen, C., 1991. Kinetics of binding of dihydropyridine calcium channel ligands to skeletal muscle membranes: evidence for low-affinity sites for the involvement of G-proteins. Biochemistry 30, 5716–5721.

Dunn, S.M.J., Bladen, C., 1992. Low-affinity binding sites for 1,4-dihydropyridines in skeletal muscle transverse tubule membranes revealed by changes in flourescence of felodopine. Biochemistry 31, 4039–4045.

Dunn, S.M.J., Bhat, M., Oz, M., 1994. In: Foa, P., Walsh, M. (Eds.), In Ion channels and Ion Transport. Springer-Verlag, New York, pp. 1–18.

Feldmeyer, D., Melzer, W., Pohl, B., Zollner, P., 1993. A possible role of sarcoplasmic Ca^{2+} release in modulating the slow Ca^{2+} current of skeletal muscle. Pflugers Arch. 425, 54–61.

Flockerzi, V., Oeken, H.J., Hofmann, F., Pelzer, D., Cavalie, A., Trautwein, W., 1986. Purified dihydropyridine-binding site from skeletal muscle T-tubules is a functional channel. Nature 323, 66–68.

Fosset, M., Jaimovich, E., Delpont, E., Lazdunski, M., 1983. [^{3}H]Nitrendipine receptors in skeletal muscle: properties and preferential localization in transverse tubules. J. Biol. Chem. 10, 6086–6092.

Franks, N.P., Lieb, W.R., 1994. Molecular and cellular mechanisms of general anaesthesia. Nature 367, 607–614.

Glossmann, H., Ferry, D., 1983. Solubilization and partial purification of putative calcium channels labeled with [^{3}H]-nimodipine. Naunyn-Schmiedeberg's Arch. Pharmacol. 323, 279–291.

Guo, J., Ikeda, S.R., 2004. Endocannabinoids modulate N-type calcium channels and G-protein-coupled inwardly rectifying potassium channels via CB1 cannabinoid receptors heterologously expressed in mammalian neurons. Mol. Pharmacol. 65, 665–674.

Habuchi, Y., Furukawa, T., Tanaka, H., Lu, L.L., Morikawa, J., Yoshimura, M., 1995. Ethanol inhibition of Ca^{2+} and Na^{+} currents in the guinea-pig heart. Eur. J. Pharmacol. 292, 143–149.

Hawtorn, M.H., Ferrante, J.N., Kwon, Y.W., Rutledge, A., Luchowski, E., Bangalore, R., Triggle, D.J., 1992. Effect of an homologous series of aliphatic alcohols on neuronal and smooth muscle voltage-dependent Ca^{2+} channels. Eur. J. Pharmacol. 229, 143–148.

Henry, D.J., Chavkin, C., 1995. Activation of inwardly rectifying potassium channels (GIRK1) by co-expressed rat brain cannabinoid receptors in *Xenopus* oocytes. Neurosci. Lett. 186, 91–94.

Hilgemann, D.W., 2004. Biochemistry. Oily barbarians breach ion channel gates. Science 304, 265–270.

Howlett, A.C., Mukhopadhyay, S., 2000. Cellular signal transduction by anandamide and 2-arachidonoylglycerol. Chem. Phys. Lipids 108, 53–70.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International Union of Pharmacology: XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.

Johnson, D.A., Heald, S.L., Dally, R.D., Janis, R.A., 1993. Isolation, identification and synthesis of an endogenous arachidonic amide that inhibits calcium channel antagonist 1,4-dihydropyridine binding. Prostaglandins, Leukot. Essent. Fat. Acids 48, 429–437.

Kumbaraci, N.M., Nastuk, W.L., 1980. Effects of delta 9-tetrahydrocannabinol on excitable membranes and neuromuscular transmission. Mol. Pharmacol. 17, 344–349.

Leaf, A., Xiao, Y.F., Kang, J.X., 2002. Interactions of *n*−3 fatty acids with ion channels in excitable tissues. Prostaglandins, Leukot. Essent. Fat. Acids 67, 1113–1120.

Lundbaek, J.A., Birn, P., Girshman, J., Hansen, A.J., Andersen, O.S., 1996. Membrane stiffness and channel function. Biochemistry 35, 3825–3830.

Maingret, F., Patel, A.J., Lazdunski, M., Honore, E., 2001. The endocannabinoid anandamide is a direct and selective blocker of the background K(+) channel TASK-1. EMBO J. 20, 47–54.

Mamas, M.A, Terrar, D.A., 2001. Actions of arachidonic acid on contractions and associated electrical activity in guinea-pig isolated ventricular myocytes. Exp. Physiol. 86, 437–449.

Meves, H., 1994. Modulation of ion channels by arachidonic acid. Prog. Neurobiol. 43, 175–186.

Morrill, J.A., Cannon, S.C., 1999. Effects of mutations causing hypokalaemic periodic paralysis on the skeletal muscle L-type Ca^{2+} channel expressed in *Xenopus laevis* oocytes. J. Physiol. 520, 321–336.

Nicholson, R.A., Liao, C., Zheng, J., David, L.S., Coyne, L., Errington, A.C., Singh, G., Lees, G., 2003. Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. Brain Res. 978, 194–204.

Oliver, D., Lien, C.C., Soom, M., Baukrowitz, T., Jonas, P., Fakler, B., 2004. Functional conversion between A-type and delayed rectifier K^{+} channels by membrane lipids. Science 304, 265–270.

Oz, M., Frank, G.B., 1991. Decrease in the size of tetanic responses by nitrendipine or by extracellular calcium ion removal without blocking twitches or action potentials in skeletal muscle. J. Pharmacol. Exp. Ther. 257, 575–581.

Oz, M., Dunn, S.M., Frank, G.B., 1992. $^{45}Ca^{2+}$ efflux studies in rabbit T-tubule membrane preparations in the range of late after potentials during tetanic contractions. Adv. Exp. Med. Biol. 311, 419–421.

Oz, M., Frank, G.B., Dunn, S.M.J., 1993. Voltage-dependent calcium flux across rabbit skeletal muscle transverse tubule membranes in the range of late after potentials. Can. J. Pharm. Physiol. 71, 518–521.

Oz, M., Melia, M.T., Soldatov, N., Abernethy, D.R., Morad, M., 1998. Functional coupling of human L-type Ca^{2+} channel and angiotensin AT1A receptor co-expressed in *Xenopus* oocytes: involvement of the carboxyl-terminal Ca^{2+} sensors. Mol. Pharmacol. 54, 1106–1112.

Oz, M., Tchugunova, Y., Dunn, S.M.J., 2000. Endogenous cannabinoid anandamide directly inhibits voltage-dependent calcium fluxes in rabbit T-tubule membrane preparations. Eur. J. Pharmacol. 404, 13–20.

Oz, M., Tchugunova, Y., Dunn, S.M.J., 2001. Direct inhibition of voltage-dependent calcium fluxes by ethanol and higher alcohols in rabbit T-tubule membrane preparations. Eur. J. Pharmacol. 418, 169–176.

Oz, M., Zhang, L., Morales, M., 2002a. Endogenous cannabinoid, anandamide acts as a non-competitive inhibitor on 5-HT3 receptor-mediated responses in *Xenopus* oocytes. Synapse 46, 150–156.

Oz, M., Dinc, M., Tchugunova, Y., Dunn, S.M.J., 2002b. Comparison of the effects of xenon and halothane on voltage-dependent Ca^{2+} fluxes in rabbit T-tubule membranes. Naunyn-Schmiedeberg's Arch. Pharmacol. 365, 413–417.

Oz, M., Tchugunova, Y., Dunn, S.M.J., 2002c. The effects of isoflurane on voltage-dependent calcium fluxes in rabbit T-tubule membranes; comparison with alcohols. Arch. Biochem. Biophys. 398, 275–283.

Oz, M., Ravindran, R., Zhang, L., Morales, M., 2003. Endogenous cannabinoid, anandamide inhibits neuronal nicotinic acethylcholine receptor-mediated responses in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 306, 1003–1010.

Oz, M., Zhang, L., Ravindran, R., Morales, M., Lupica, C.R., 2004a. Direct and differential effects of cannabinoid receptor ligands on α_7-nicotinic receptor-mediated currents in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 310, 1152–1160.

Oz, M., Spivak, C.E., Lupica, C.R., 2004b. The solubilizing detergents, Tween 80 and Triton X-100 non-competitively inhibit α7-nicotinic acetylcholine receptor function in *Xenopus* oocytes. J. Neurosci. Methods 137, 167–173.

Peoples, R.W., Chaoying, L., Weight, F.F., 1996. Lipid vs. protein theories of alcohol action in the nervous system. Annu. Rev. Pharmacol. Toxicol. 36, 185–201.

Piomelli, D., 2003. The molecular logic of endocannabinoid signalling. Nat. Rev., Neurosci. 4, 873–884.

Poling, J.S., Rogawski, M.A., Salem, N., Vicini, S., 1996. Anandamide, an endogenous cannabinoid inhibits Shaker-related voltage-gated K^+ channels. Neuropharmacology 35, 983–991.

Rosemblatt, M.S., Scales, D.J., 1989. Morphological, immunological and biochemical characterization of purified transverse tubule membranes isolated from rabbit skeletal muscle. Mol. Cell. Biochem. 87, 57–69.

Shiamsue, K., Urushidani, T., Hagiwara, M., Nagao, T., 1996. Effects of anandamide and arachidonic acid on specific binding of (+)-PN200–110, diltiazem and (−)-desmethoxyverapamil to L-type Ca^{2+} channel. Eur. J. Pharmacol. 296, 347–350.

Shimada, T., Somlyo, A.P., 1992. Modulation of voltage-dependent Ca channel current by arachidonic acid and other long-chain fatty acids in rabbit intestinal smooth muscle. J. Gen. Physiol. 100, 27–44.

Soldatov, N., Oz, M., O'Brien, K.A., Abernethy, D.R., Morad, M., 1998. Molecular determinants of L-type Ca^{2+} channel inactivation. J. Biol. Chem. 273, 957–963.

Xiao, Y.F., Gomez, A.M., Morgan, J.P., Lederer, W.J., Leaf, A., 1997. Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. Proc. Natl. Acad. Sci. U. S. A. 94, 4182–4187.

Venance, L., Piomelli, D., Glowinski, J., Giaume, C., 1995. Inhibition by anandamide of gap junctions and intracellular calcium signaling in striatal astrocytes. Nature 376, 590–594.

Vreugdenhil, M., Bruehl, C., Voskuyl, R.A., Kang, J.X., Leaf, A., Wadman, W.J., 1996. Polyunsaturated fatty acids modulate sodium and calcium currents in CA1 neurons. Proc. Natl. Acad. Sci. U. S. A. 93, 12559–12563.